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# Mitochondrial, but not rDNA, genes fail to discriminate dinoflagellate species in the genus *Ostreopsis*

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ABSTRACT

The marine dinoflagellate genus *Ostreopsis* includes species producing potent toxic compounds, such as paliytoxin and palytoxin analogs, which might cause problematic blooms in the Mediterranean Sea and other tropical or temperate areas. Phylogenetic and phylogeographical studies based on sequences of ribosomal genes, revealed the existence of distinct species and, within them, clades in relation to geographical distribution potentially representing new or cryptic species. The morphological variability of *Ostreopsis* complicates the identification of species; thus, molecular analyses of isolates or field samples can be helpful. The aim of this study was to improve the characterization of *Ostreopsis* species and investigate the geographical distribution by using large dataset composed of both new and old sampled isolates. To determine if mitochondrial genes can be used to identify *Ostreopsis* species, we designed new primers sets then amplified and sequenced representative regions of the COI (cytochrome c oxydase 1) and cob (cytochrome b) genes. Phylogenetic analyses of the resulting mitochondrial DNA (mtDNA) and existing or new ribosomal DNA (rDNA) sequence data showed little divergence in the mtDNA sequences among *Ostreopsis* species indicating that neither the COI or cob genes are phylogenetically informative. In contrast, the ribosomal gene phylogeny indicated the existence of distinct *Ostreopsis* species. A network of haplotypes (based on ITS-5.8S rDNA) inferred from *O.* cf. *ovata* isolates collected worldwide revealed that Atlantic/Mediterranean and Indo/Pacific areas might host two separated large populations.

In conclusion, it appears that rDNA gene sequences provide an effective molecular means of distinguishing the phylogenetic and phylogeographical relationships among *Ostreopsis* species.



## 1. Introduction

*Ostreopsis* Schmidt is an epi-benthic dinoflagellate genus, found in tropical and temperate coastal waters worldwide (Pin et al., 2001; Shears and Ross, 2009). Some species are no-toxic (Ciminiello et al., 2013), but others are of concern because they produce potent non protein toxins, including palytoxin-like compounds and various ovatoxins (Ukena et al., 2001; Riobó et al., 2006; Ciminiello et al., 2012; Uchida et al., 2013). In the Mediterranean Sea, in particular, blooms of *O.* cf. *ovata* have become increasingly frequent and are associated with benthic invertebrate mortalities, accumulation of toxins in filter-feeding organisms and human intoxication due to lysed cells and the formation of toxic aerosols along the beaches (Gallitelli et al., 2005; Tichadou et al., 2010). The ability to accurately identify whether toxic or non-toxic species are present is therefore important for assessing potential ecological or public health risks (Aligizaki et al., 2011; Privitera et al., 2012; Vila et al., 2012). Unfortunately, *Ostreopsis* species are morphologically plastic and nearly impossible to discriminate using light or scanning electron microscopy with the current criteria in use (Guerrini et al., 2010; Selina and Orlova, 2010; Honsell et al., 2011; Accoroni et al., 2012; Tawong et al., 2014) and a taxonomic revision of the whole genus is necessary based on new criteria. In contrast, *Ostreopsis* species can be readily distinguished according to various rDNA gene regions (Penna et al., 2005; Battocchi et al., 2010; Laza-Martinez et al.,

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1568-9883/

2011; Nascimento et al., 2012). For example, phylogeographical studies based on different single and/or concatenated ribosomal genes from the Mediterranean Sea, Atlantic Ocean, Pacific areas, Japan and New Zealand revealed that the sequences belong to well-defined clades which correspond to different species or groups (Penna et al., 2010; Sato et al., 2011; David et al., 2013). More recently, as more isolates from Atlantic, Mediterranean Sea, and Pacific regions have been sequenced, distinct subclades have become apparent. These distinct subclades likely correspond with morphologically cryptic species or highly divergent populations. In other dinoflagellate groups, the divergences observed in the *Ostreopsis* ITS-5.8S and LSU subclades would be consistent with species level divergences (Litaker et al., 2007; Penna et al., 2012; Wang et al., 2014). Actually, defining *Ostreopsis* based solely on these molecular criteria however, has yet to be formally proposed and accepted. Consequently, isolates, such as those morphologically assigned to *O.* cf. *ovata*, are currently referred to as putative species complex (Sato et al., 2011; Kang et al., 2013; Tawong et al., 2014). Again, extensive morphological studies have failed to reveal morphologically informative characters or other chemotaxonomic features which could be used to consistently discriminate closely related species. In this study, we examined whether mitochondrial genes, such as COI (cytochrome *c* oxidase) and cob (cytochrome b), obtained from geographically dispersed locations would prove helpful in genetically defining *Ostreopsis* species. Mitochondrial genes have proven capable of differentiating species in a wide range of organisms including birds, mammals, or macroalgae (Coyer et al., 2006; Francis et al., 2010). Mitochondrial genes in some slowly evolving lineages, such as within certain vascular plant lineages, however, lack sufficient sequence variability to discriminate among closely related species (Kress et al., 2005; Lahaye et al., 2008). The usefulness of mitochondrial genes to infer protist phylogeny is still under investigation with an increasing number of studies on marine species. To date the COI gene has proved a useful marker to discriminate species within a genus, such as the diatom *Sellaphora* (Evans et al., 2007). Within marine dinoflagellates the power of mtDNA genes to discriminate species varies depending on the specific taxa analyzed (Lin et al., 2009). Both COI and cob genes seem to evolve at vastly different rates in the different dinoflagellate lineages. In some cases, such as Prorocentrales, the sequence variation is sufficient to inform taxonomic questions ranging from species level divergences to the phylogenetic positions for some major dinoflagellate groups. Within other taxa, such as other Gonyaulacales and Kareniaceae, the mitochondrial genes failed to provide phylogenetically information with *Alexandrium* and *Karenia*, respectively (Zhang et al., 2007; Murray et al., 2009; Stern et al., 2010).

The numerous isolates sequenced in this study significantly increased the mitochondrial *Ostreopsis* sequences available for analysis compared with previous studies (Penna et al., 2005, 2010). The resulting phylogeny was compared to those based on aligned ITS-5.8S and LSU rDNA sequences obtained from this study and GenBank to determine if the phylogenetic and phylogeographical patterns in the mitochondrial gene phylogeny are more informative than those based on rDNA sequences. The combined data where used to make recommendations on putative species within the genus *Ostreopsis*.

## 2. Materials and methods

### 2.1. Microalgal cultures and sample collection

The *Ostreopsis* spp. isolates used in this study are listed in Tables 1 and 2 and S1 (see also below). *Ostreopsis* spp. strains were isolated by micropipetting from seawater, net and macroalgal samples collected from several localities of the Mediterranean Sea, Western and Eastern Atlantic Ocean, Indian and Pacific Ocean from 2000 to 2010. Clonal cultures were established and maintained in F/4-Si medium (Guillard, 1975) at a temperature of 23 ± 1 °C. Light was provided by cool-white fluorescent bulbs (photon flux of 100 μE $m^{-2}$ $s^{-1}$) with a standard 14:10 h light–dark cycle. Subsamples of cultures (10 mL) were collected at the exponential growth phase by centrifugation at 4000 × *g* for 15 min, at room temperature. The supernatant was removed and the pellets were immediately processed or stored at −80 °C until DNA extraction.

### 2.2. DNA extraction

Genomic DNA was extracted from cultures in exponential growth phase, or from pellets stored at −80 °C, using the DNeasy Plant Kit (Qiagen, Valencia, CA, USA). Purified total DNA was quantified using a Qubit fluorometer with a Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbard, CA, USA). Alternatively, genomic DNA was quantified on agarose gel using serially diluted DNA Marker Lambda (MBI Fermentas, Germany) and a gel-doc apparatus (Bio-Rad, Hercules, CA, USA).

### 2.3. Mitochondrial gene primer design

A new set of genus-specific primers was designed based on *Ostreopsis* spp. and other related dinoflagellate COI and cob consensus gene sequences available from GenBank. The sequence alignment was performed with ClustalW v. 2.0 (Larkin et al., 2007) and primers were designed using OLIGO Primer Analysis Software ver 6.65 (Molecular Biology Insights, Inc., Cascade, CO, USA). The genus-specific primers for the amplification of 715 bp of COI were OstrCoxF (5′-TTG GGA TCT CCG GTA CA-3′) and OstrCoxR (5′-AAA TAC AGC TCA TGG CAA AG-3′), while the genus specific primers for the amplification of 730 bp cob were OstrCobF (5′-TNA TAN ATC AGA TCT TAN TTC AGC AT-3′), and OstrCobR (5′-AAR GKA AGA CNN NCA AAG ATG TTA G-3′). The primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

### 2.4. Amplification and sequencing of mitochondrial and ribosomal genes

PCR amplification of COI and cob genes was as follows: tubes contained 25–50 μL of mixture of 400–600 μM of dNTPs, 0.2–0.6 μM of each primer, 3–6 mM of $MgCl_2$, 1× reaction buffer (Diatheva, Italy), 0.4–0.8 μg $\mu L^{-1}$ BSA (bovine serum albumine, Sigma), 0.2 U Taq polymerase (Diatheva, Italy) and 5 ng of DNA template. PCR conditions were as follows: an initial denaturation at 95 °C for 10 min, 35 cycles of 30 s at 95 °C, 30 s at 52–56 °C, and 90 s at 72 °C and a final extension step of 7 min at 72 °C. PCR amplifications of 5.8S-ITS and LSU (D1/D2 domains) ribosomal genes has been reported in Penna et al. (2008) and Fraga et al. (2008), respectively. All PCR amplified products were purified using DNeasy gel extraction Kit (Qiagen, Valencia, CA, USA), and the products were directly sequenced (Eurofins MWG Operon, Ebersberg, Germany). The sequences of mitochondrial and ribosomal genes obtained from new *Ostreopsis* spp. cultured isolates were listed in Tables 1 and 2, respectively. All sequences were deposited in GenBank and EMBL-EBI-ENA. The other ribosomal sequences from *Ostreopsis* spp. isolates included in this study are listed in Table S1.

Supplementary Table S1 related to this article can be found, in the online version, at doi:10.1016/j.hal.2014.10.004.

### 2.5. Phylogenetic and molecular analyses

The COI and cob mtDNA and ITS-5.8S and LSU rDNA sequences obtained as described above were aligned using ClustalW v. 2.0. Ambiguously aligned positions and divergent regions were

**Table 1**
List of new (from 2008) or other (bold) *Ostreopsis* spp. isolates, sampling locations, isolator, and mitochondrial COI and cob GenBank accession numbers, and original source.

| Species | Strain ID | Geographical origin and collecting period | Isolator | Accession no. COI | Accession no. cob | Source |
|---|---|---|---|---|---|---|
| *O.* cf. *ovata* | An08 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2008 | Penna A. | JX065433 | | This study |
| *O.* cf. *ovata* | CBA166 | Trieste, Italy, Adriatic Sea, Mediterranean, 2009 | Penna A. | JX065438 | – | This study |
| *O.* cf. *ovata* | CBA1704 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Penna A. | JX065434 | JX065512 | This study |
| *O.* cf. *ovata* | CBA1711 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Penna A. | JX065433 | – | This study |
| *O.* cf. *ovata* | CBA1714 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Penna A. | JX065434 | – | This study |
| *O.* cf. *ovata* | CBA1722 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Battocchi C. | JX065435 | – | This study |
| *O.* cf. *ovata* | CBA1729 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Battocchi C. | JX065436 | – | This study |
| *O.* cf. *ovata* | CBA1746 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Capellacci S. | JX065437 | – | This study |
| *O.* cf. *ovata* | CBA1747 | Portonovo, Italy, Adriatic Sea, Mediterranean, 2010 | Capellacci S. | JX065432 | – | This study |
| *O.* cf. *ovata* | 00AB08 | Bari, Italy, Adriatic Sea, Mediterranean, 2008 | Guerrini F. | JX065506 | – | This study |
| *O.* cf. *ovata* | CBA1399 | Bari, Italy, Adriatic Sea, Mediterranean, 2009 | Capellacci S. | JX065532 | – | This study |
| *O.* cf. *ovata* | TAO08 | Taormina, Italy, Ionian Sea, Mediterranean, 2008 | Battocchi C. | JX065545 | – | This study |
| *O.* cf. *ovata* | CBA1291 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Casabianca S. | JX065483 | – | This study |
| *O.* cf. *ovata* | CBA1808 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065589 | JX065522 | This study |
| *O.* cf. *ovata* | CBA1823 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065484 | JX065523 | This study |
| *O.* cf. *ovata* | CBA1829 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065485 | – | This study |
| *O.* cf. *ovata* | CBA1845 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065486 | – | This study |
| *O.* cf. *ovata* | CBA1848 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065487 | – | This study |
| *O.* cf. *ovata* | CBA1849 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065488 | – | This study |
| *O.* cf. *ovata* | CBA1851 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065489 | – | This study |
| *O.* cf. *ovata* | CBA1437 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Battocchi C. | JX065476 | JX065518 | This study |
| *O.* cf. *ovata* | CBA1461 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Battocchi C. | JX065477 | JX065519 | This study |
| *O.* cf. *ovata* | CBA1463 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Battocchi C. | JX065478 | – | This study |
| *O.* cf. *ovata* | CBA1472 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Battocchi C. | JX065479 | – | This study |
| *O.* cf. *ovata* | CBA1479 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065482 | JX065520 | This study |
| *O.* cf. *ovata* | CBA1485 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065480 | JX065521 | This study |
| *O.* cf. *ovata* | CBA1649 | Porto Romano, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Battocchi C. | JX065481 | – | This study |
| *O.* cf. *ovata* | Massa08 | Marina di Massa, Italy, Thyrrenian Sea, Mediterranean, 2008 | Battocchi C. | JX065546 | – | This study |
| *O.* cf. *ovata* | CBA1319 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065464 | – | This study |
| *O.* cf. *ovata* | CBA 1323 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065465 | – | This study |
| *O.* cf. *ovata* | CBA1586 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065466 | – | This study |
| *O.* cf. *ovata* | CBA 1587 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065467 | – | This study |
| *O.* cf. *ovata* | CBA 1588 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065468 | – | This study |
| *O.* cf. *ovata* | CBA1590 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065469 | – | This study |
| *O.* cf. *ovata* | CBA1592 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065470 | – | This study |
| *O.* cf. *ovata* | CBA1597 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065471 | JX065516 | This study |
| *O.* cf. *ovata* | CBA1598 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065472 | – | This study |
| *O.* cf. *ovata* | CBA1599 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065473 | – | This study |
| *O.* cf. *ovata* | CBA1600 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065474 | – | This study |
| *O.* cf. *ovata* | CBA1601 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065475 | JX065517 | This study |
| *O.* cf. *ovata* | CBA1502 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065445 | JX065509 | This study |
| *O.* cf. *ovata* | CBA1505 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065439 | – | This study |
| *O.* cf. *ovata* | CBA1509 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065440 | – | This study |
| *O.* cf. *ovata* | CBA1517 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065441 | JX065510 | This study |
| *O.* cf. *ovata* | CBA 1518 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065442 | – | This study |
| *O.* cf. *ovata* | CBA1526 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065443 | JX065511 | This study |
| *O.* cf. *ovata* | CBA1617 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065444 | – | This study |
| *O.* cf. *ovata* | CBA1218 | La Spezia, Italy, Ligurian Sea, Mediterranean, 2009 | Casabianca S. | JX065533 | – | This study |
| *O.* cf. *ovata* | CBA1242 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Capellacci S. | JX065446 | – | This study |
| *O.* cf. *ovata* | CBA1244 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Capellacci S. | JX065447 | – | This study |
| *O.* cf. *ovata* | CBA1245 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Capellacci S. | JX065448 | – | This study |
| *O.* cf. *ovata* | CBA1412 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Capellacci S. | JX065449 | – | This study |
| *O.* cf. *ovata* | CBA1420 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065450 | – | This study |
| *O.* cf. *ovata* | CBA1421 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065451 | – | This study |
| *O.* cf. *ovata* | CBA1427 | Genova, Italy, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065452 | – | This study |
| *O.* cf. *ovata* | CBA1553 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065490 | JX065524 | This study |
| *O.* cf. *ovata* | CBA1556 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065491 | JX065525 | This study |
| *O.* cf. *ovata* | CBA1563 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065492 | – | This study |
| *O.* cf. *ovata* | CBA1570 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065493 | – | This study |
| *O.* cf. *ovata* | CBA1574 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065494 | JX065526 | This study |
| *O.* cf. *ovata* | CBA1581 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065495 | – | This study |
| *O.* cf. *ovata* | CBA1628 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065496 | JX065527 | This study |
| *O.* cf. *ovata* | CBA1631 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065497 | – | This study |
| *O.* cf. *ovata* | CBA1633 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065498 | – | This study |
| *O.* cf. *ovata* | CBA1636 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065499 | – | This study |
| *O.* cf. *ovata* | CBA1637 | Villefranche, France, Ligurian Sea, Mediterranean, 2010 | Battocchi C. | JX065500 | – | This study |
| *O.* cf. *ovata* | **VGO820** | Tossa del Mar, Spain, Catalan Sea, Mediterranean, 2005 | Fraga S. | JX065501 | JX065541 | Penna et al. (2010) |
| *O.* cf. *ovata* | VGO960 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Fraga S. | JX065503 | JX065542 | This study |
| *O.* cf. *ovata* | VGO964 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Fraga S. | JX065504 | – | This study |
| *O.* cf. *ovata* | CBA1661 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Battocchi C. | JX065458 | – | This study |
| *O.* cf. *ovata* | CBA1662 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Battocchi C. | JX065459 | – | This study |
| *O.* cf. *ovata* | CBA1663 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Battocchi C. | JX065460 | – | This study |
| *O.* cf. *ovata* | CBA1667 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Battocchi C. | JX065461 | – | This study |
| *O.* cf. *ovata* | CBA1684 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Battocchi C. | JX065462 | – | This study |

**Table 1** (*Continued*)

| Species | Strain ID | Geographical origin and collecting period | Isolator | Accession no. COI | Accession no. cob | Source |
|---|---|---|---|---|---|---|
| *O.* cf. *ovata* | CBA1693 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Battocchi C. | JX065463 | – | This study |
| *O.* cf. *ovata* | **CNR-Z1** | Paguera, Spain, Balearic Sea, Mediterranean, 2001 | Giacobbe M.G. | JX065502 | JX065536 | Penna et al. (2005) |
| *O.* cf. *ovata* | **KC70** | Athens, Greece, Aegean Sea, Mediterranean, 2007 | Aligizaki K. | JX065507 | JX065537 | Penna et al. (2010) |
| *O.* cf. *ovata* | **KC71** | Athens, Greece, Aegean Sea, Mediterranean, 2007 | Aligizaki K. | JX065508 | JX065538 | Penna et al. (2010) |
| *O.* cf. *ovata* | CBA1759 | Aggelochori, Greece, Aegean Sea, Mediterranean, 2010 | Battocchi C. | JX065453 | JX065513 | This study |
| *O.* cf. *ovata* | CBA1770 | Aggelochori, Greece, Aegean Sea, Mediterranean, 2010 | Battocchi C. | JX065454 | JX065514 | This study |
| *O.* cf. *ovata* | CBA1774 | Aggelochori, Greece, Aegean Sea, Mediterranean, 2010 | Battocchi C. | JX065455 | – | This study |
| *O.* cf. *ovata* | CBA1782 | Aggelochori, Greece, Aegean Sea, Mediterranean, 2010 | Battocchi C. | JX065456 | JX065515 | This study |
| *O.* cf. *ovata* | CBA1797 | Aggelochori, Greece, Aegean Sea, Mediterranean, 2010 | Battocchi C. | JX065457 | – | This study |
| *O.* cf. *ovata* | VGO1001 | Famara, Canary Isl., Spain, E Atlantic Ocean, 2008 | Rodriguez F. | JX065505 | JX065543 | This study |
| *O.* cf. *ovata* | **OS13BR** | Rio de Janeiro, Brazil, W Atlantic Ocean, 2000 | Fraga S. | JX065528 | – | Penna et al. (2010) |
| *O.* cf. *ovata* | **OS16BR** | Rio de Janeiro, Brazil, W Atlantic Ocean, 2000 | Fraga S. | JX065529 | – | Penna et al. (2010) |
| *O.* cf. *ovata* | **OS20BR** | Rio de Janeiro, Brazil, W Atlantic Ocean, 2000 | Fraga S. | JX065530 | – | Penna et al. (2010) |
| *O.* cf. *ovata* | LCH001 | Thua Thien-Hue, Vietnam, South China Sea, Pacific, 2009 | Nguyen N.L. | JX065572 | – | This study |
| *O.* cf. *ovata* | QB04 | Quang Binh, Vietnam, South China Sea, Pacific, 2009 | Nguyen N.L. | JX065573 | JX065580 | This study |
| *O.* cf. *ovata* | VGO1056 | Belize, Caribbean Sea, N Atlantic Ocean, 2009 | Holland C. | JX065577 | JX065581 | This study |
| *O.* cf. *siamensis* | IO-9601 | Sines, Portugal, E Atlantic, 2008 | Veloso V. | JX065578 | – | This study |
| *O.* cf. *siamensis* | IO-9602 | Sines, Portugal, E Atlantic, 2008 | Veloso V. | – | JX065582 | This study |
| *O.* cf. *siamensis* | IO-9604 | Cascais, Portugal, E Atlantic, 2010 | Veloso V. | JX065579 | JX065583 | This study |
| *O.* cf. *lenticularis* | NT011 | Ninh Thuan, Vietnam, South China Sea, 2009 | Nguyen N.L. | JX065574 | – | This study |
| *O.* cf. *lenticularis* | NT012 | Ninh Thuan, Vietnam, South China Sea, 2009 | Nguyen N.L. | JX065575 | – | This study |
| *O.* cf. *lenticularis* | NT013 | Ninh Thuan, Vietnam, South China Sea, 2009 | Nguyen N.L. | JX065576 | – | This study |
| *Ostreopsis* spp. | KC84 | Cyprus, Aegean Sea, Mediterranean, 2008 | Aligizaki K. | JX065577 | JX065539 | This study |
| *Ostreopsis* spp. | KC86 | Crete, Greece, Aegean Sea, Mediterranean, 2009 | Aligizaki K. | JX065578 | JX065540 | This study |
| *Ostreopsis* spp. | **VGO881** | Lanzarote, Canary Isl., Spain, E Atlantic Ocean, 2005 | Fraga S. | JX065531 | – | This study, |
| *Ostreopsis* spp. | CBA0203 | Honolulu, N Pacific Ocean, Hawaii, USA, 2010 | Capellacci S. | JX065579 | JX065590 | This study |

**Table 2**
List of new *Ostreopsis* spp. isolates, sampling locations, isolator, ITS – 5.8S and LSU gene sequence accession numbers from GenBank and EMBL.

| Species | Strain ID | Geographical origin and collecting period | Isolator | Accession no. ITS-5.8S | Accession no. LSU |
|---|---|---|---|---|---|
| *O.* cf. *ovata* | CBA166 | Trieste, Italy, Adriatic Sea, Mediterranean, 2009 | Penna A. | JX065557 | |
| *O.* cf. *ovata* | CBA1823 | Taormina, Italy, Ionian Sea, Mediterranean, 2010 | Battocchi C. | JX065555 | JX065564 |
| *O.* cf. *ovata* | CBA1597 | Marina di Pisa, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Casabianca S. | JX065554 | JX065563 |
| *O.* cf. *ovata* | CBA1502 | Alghero, Italy, Tyrrhenian Sea, Mediterranean, 2010 | Capellacci S. | JX065553 | JX065562 |
| *O.* cf. *ovata* | CBA1553 | Villefranche, Ligurian Sea, Mediterranean, France, 2010 | Battocchi C. | JX065556 | JX065565 |
| *O.* cf. *ovata* | VGO960 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Fraga S. | – | JX065567 |
| *O.* cf. *ovata* | VGO964 | Llavaneres, Spain, Catalan Sea, Mediterranean, 2008 | Fraga S. | JX065552 | JX065566 |
| *O.* cf. *ovata* | VGO1001 | Famara, Canary Island, Spain, E Atlantic Ocean, 2008 | Rodriguez F. | JX065551 | JX065560 |
| *O.* cf. *ovata* | LCH001 | Thua Thien-Hue, Vietnam, South China Sea, Pacific, 2009 | Nguyen N.L. | – | JX065569 |
| *O.* cf. *ovata* | QB04 | Quang Binh, Vietnam, South China Sea, Pacific, 2009 | Nguyen N.L. | – | JX065571 |
| *O.* cf. *ovata* | QB03 | Quang Binh, Vietnam, South China Sea, Pacific, 2009 | Nguyen N.L. | – | KC900890 |
| *O.* cf. *ovata* | QB06 | Quang Binh, Vietnam, South China Sea, Pacific, 2009 | Nguyen N.L. | – | KC900891 |
| *O.* cf. *ovata* | VGO1056 | Belize, Caribbean Sea, N Atlantic Ocean, 2009 | Holland C. | JX065586 | JX065588 |
| *O.* cf. *siamensis* | IO-9601 | Sines, Portugal, E Atlantic, 2008 | Veloso V. | JX065587 | – |
| *O.* cf. *lenticularis* | NT011 | Ninh Thuan, Vietnam, South China Sea, 2009 | Nguyen N.L. | JX065584 | – |
| *O.* cf. *lenticularis* | NT012 | Ninh Thuan, Vietnam, South China Sea, 2009 | Nguyen N.L. | JX065585 | – |
| *O.* cf. *lenticularis* | NT013 | Ninh Thuan, Vietnam, South China Sea, 2009 | Nguyen N.L. | – | JX065570 |
| *Ostreopsis* spp. | KC84 | Cyprus, Aegean Sea, Mediterranean, 2008 | Aligizaki K. | JX065549 | JX065558 |
| *Ostreopsis* spp. | KC86 | Crete, Greece, Aegean Sea, Mediterranean, 2009 | Aligizaki K. | JX065550 | JX065559 |
| *Ostreopsis* spp. | CBA0203 | Honolulu, Hawaii, N Pacific Ocean, USA, 2010 | Capellacci S. | JX065552 | JX065561 |

excluded from the alignment by using software Gblocks (http://molevol.cmima.csic.es/castresana/Gblocks.html) with default settings. Analyses were conducted on ITS-5.8S, LSU, COI and cob sequence alignments, separately.

The jModelTest v. 0.1.1 (Posada, 2008) was used to determine the evolutionary model that best fitted the data according to the corrected Akaike Information Criterion. For the *Ostreopsis* ITS-5.8S rDNA alignment the most appropriate evolutionary model was found to be a HKY with a proportion of invariable sites (I), while for LSU ribosomal gene a general time reversible model (GTR) with a gamma distributed rate of variation among sites was selected. The most appropriate evolutionary model for *Ostreopsis* cob gene sequence alignment was F81 with a proportion of invariable sites (I), while F81 with a gamma distributed rate of variation among sites ($\Gamma$) was selected for *Ostreopsis* COI alignment. The shape parameter $\alpha$ of the gamma distribution ($\Gamma$) and the proportion of invariable sites ($I$) were estimated from the dataset using default options. Neighbor-Joining (NJ) analyses were performed using Phylip v. 3.69 (Felsenstein, 1989). The robustness of NJ trees was tested by bootstrapping using 1000 pseudo-replicates.

Maximum-Likelihood (ML) analyses were run with PhyMl v. 3.0 (Guindon et al., 2010). Bootstrap values were calculated with 1000 pseudo-replicates.

Bayesian analyses (BI) were performed using MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) with the following settings: four Markov chains were run for two millions of generations with a sampling frequency of 100 generations, resulting in 20,000 sampled trees. Each analysis used default priors and was repeated twice starting from independent points. The last 15,000 trees were used to estimate Bayesian posterior probabilities, while the first 5000 were discarded as burn-in. Majority-rule consensus trees containing the posterior probabilities were constructed based on the results of two independent runs.

The sequences of *Alexandrium catenella* JF266633, EF178143, AB290123 and AB290124 were used as outgroup for the *Ostreopsis* ITS-5.8S, LSU, COI and cob, gene phylogenetic analyses, respectively.

Statistical parsimony network (Templeton et al., 1992) were obtained based on ITS-5.8S rDNA sequences of the worldwide *Ostreopsis* spp. isolates using TCS ver. 1.15 software (Clement et al., 2000).

### 2.6. Molecular statistical analysis

Average pair-wise distances (PWD) both at inter and intra-species level, based on the ITS-5.8S, LSU, COI and cob genes, were calculated with MEGA ver. 5.05 (Tamura et al., 2011) using uncorrected p-distance model including transition and transversion substitutions and homogeneous lineage pattern with uniform site rate.

## 3. Results

### 3.1. Ostreopsis COI and cob primers

Primers already published for both COI and cob genes (Zhang et al., 2007; Kamikawa et al., 2008) were tested. None of the primer set allowed 100% amplification and sequencing any of the *Ostreopsis* isolates (Table S2). Therefore, new set of COI and cob primers specific for the genus *Ostreopsis* were designed. These new primer pairs correctly amplified all our *Ostreopsis* isolates providing amplified products of the expected size (730 bp), and allowed to sequence mitochondrial genes.

Supplementary Table S2 related to this article can be found, in the online version, at doi:10.1016/j.hal.2014.10.004.

### 3.2. Phylogenetic analyses of Ostreopsis COI and cob genes

The final alignments of *Ostreopsis* spp. mitochondrial gene sequences (COI and cob) with *Alexandrium catenella* as outgroup were as follows: COI was 708 bp in length ($A$ = 28%, $T$ = 39.3%, $C$ = 18.3% and $G$ = 14.3%), with 25 polymorphic sites and a transition/transversion ratio of 0.75; cob was 705 bp in length ($A$ = 24.6%, $T$ = 43.9%, $C$ = 18.2% and $G$ = 13.3%), with 20 polymorphic sites and a transition/transversion ratio of 0.4.

Based on single COI and cob mitochondrial genes of *Ostreopsis* spp. isolates from Mediterranean Sea and worldwide, almost identical tree topologies were obtained by the NJ, ML and BI methods; therefore, only ML phylogenetic trees are presented (Fig. 1). A total of 99 COI sequences of *Ostreopsis* isolates were analyzed. The COI phylogeny showed a single clade including all *Ostreopsis* spp. isolates. This group comprised *Ostreopsis* cf. *ovata*, *Ostreopsis* cf. *siamensis*, *Ostreopsis* cf. *lenticularis* and other *Ostreopsis* spp. isolates, as VGO881, KC84, KC86 and CBA0203. The majority of the *Ostreopsis* spp. isolates from the Mediterranean Sea, Brazil and Pacific Asia shared identical sequences with exception of some isolates ($n$ = 15), which showed few nucleotide differences.

The cob mitochondrial phylogeny, based on 32 sequence isolates, showed slightly higher differences in tree topology compared to phylogenetic analysis based on COI gene, and the distinct *Ostreopsis* species were not substantially resolved.

### 3.3. Phylogenetic analyses of Ostreopsis ITS-5.8S and LSU ribosomal genes

The final alignment of *Ostreopsis* spp. ribosomal gene sequences (ITS-5.8S and LSU) with *Alexandrium catenella* as outgroup was as follows: ITS-5.8S was 390 bp in length ($A$ = 26.4%, $T$ = 34.6%, $C$ = 18.7% and $G$ = 20.2%) with 259 polymorphic sites and a transition/transversion ratio of 1.3; LSU D1/D2 domain was 692 bp in length ($A$ = 28.3%, $T$ = 32%, $C$ = 17.8% and $G$ = 21.8%) with 472 polymorphic sites and a transition/transversion ratio of 1.3.

Based on single ITS-5.8S and LSU rDNA sequences only minor differences among NJ, ML and BI analyses were found; therefore, only ML phylogenetic trees are presented (Fig. 2). The ITS-5.8S rDNA phylogeny, based on 72 isolates of *Ostreopsis* spp., identified four major clades within the genus *Ostreopsis*: the first comprising *Ostreopsis* spp. CBA0203 from Hawaii, and strains identified as *Ostreopsis* cf. *labens* and *Ostreopsis* cf. *lenticularis* from Pacific Ocean; the second clade comprised the *Ostreopsis* spp. VGO881 from the Atlantic Ocean (Canary Islands) with *Ostreopsis* spp. KC84 and KC86, from Mediterranean Sea (Crete and Cyprus, respectively); the third clade grouped all *Ostreopsis* cf. *siamensis* from Mediterranean Sea and East Atlantic Ocean; finally, the fourth grouping included all *Ostreopsis* cf. *ovata* from the Mediterranean Sea, Atlantic and Indo-Pacific Oceans. All these clusters were strongly supported by high bootstrap and posterior probability values. Furthermore, within this latter lineage three well-resolved clades were identified: the first group included all the *O.* cf. *ovata* from Mediterranean Sea, Atlantic and Pacific Ocean; the second consisted of isolates of *O.* cf. *ovata* from Indian Ocean (Malaysia), Pacific Ocean (Indonesia) and Atlantic Ocean (Caribbean Sea); the third comprised all the *O.* cf. *ovata* only from Pacific Ocean, as Malaysia (South China Sea), Japan Sea and Cook Island. The Mediterranean, Atlantic and west Pacific *O.* cf. *ovata* isolates shared the same sequences.

The LSU rDNA phylogeny that was obtained from 49 isolates of *Ostreopsis* spp., showed some differences in tree topology compared with 5.8S-ITS rDNA analysis. Indeed, the first split from the outgroup was constituted by a clade including the *Ostreopsis* spp. KC84, KC86 and VGO881 isolates, and a clade that comprised two sub-clades of *Ostreopsis* cf. *lenticularis* from S. China Sea (Vietnam and Malaysia, respectively), along with the *Ostreopsis* spp. CBA0203 from Hawaii. The second clade grouped the *Ostreopsis* cf. *siamensis* from Mediterranean Sea and the thirth clade was comprised of all the *Ostreopsis* cf. *ovata* isolates collected from worldwide. All these lineages were strongly supported by high bootstrap and posterior probability values.

### 3.4. Network analysis of the ITS-5.8S ribosomal gene

A haplotype network analysis of the ITS-5.8S rDNA from *Ostreopsis* cf. *ovata* isolates originating from the Mediterranean, Atlantic and Pacific regions was undertaken to determine whether *O.* cf. *ovata* as currently described represents a collection of distinct cryptic species (Fig. 3). Nucleotide sequences were aligned to form a 356 bp fragment. A total of 13 haplotypes and 106 polymorphic sites were found, with 108 substitutions (transversion/transition ratio of 1.07) and 23 indels. Within the *O.* cf. *ovata* the different haplotypes were separated by a maximum of 50 mutational steps from the most frequent one, CBA166, which was found

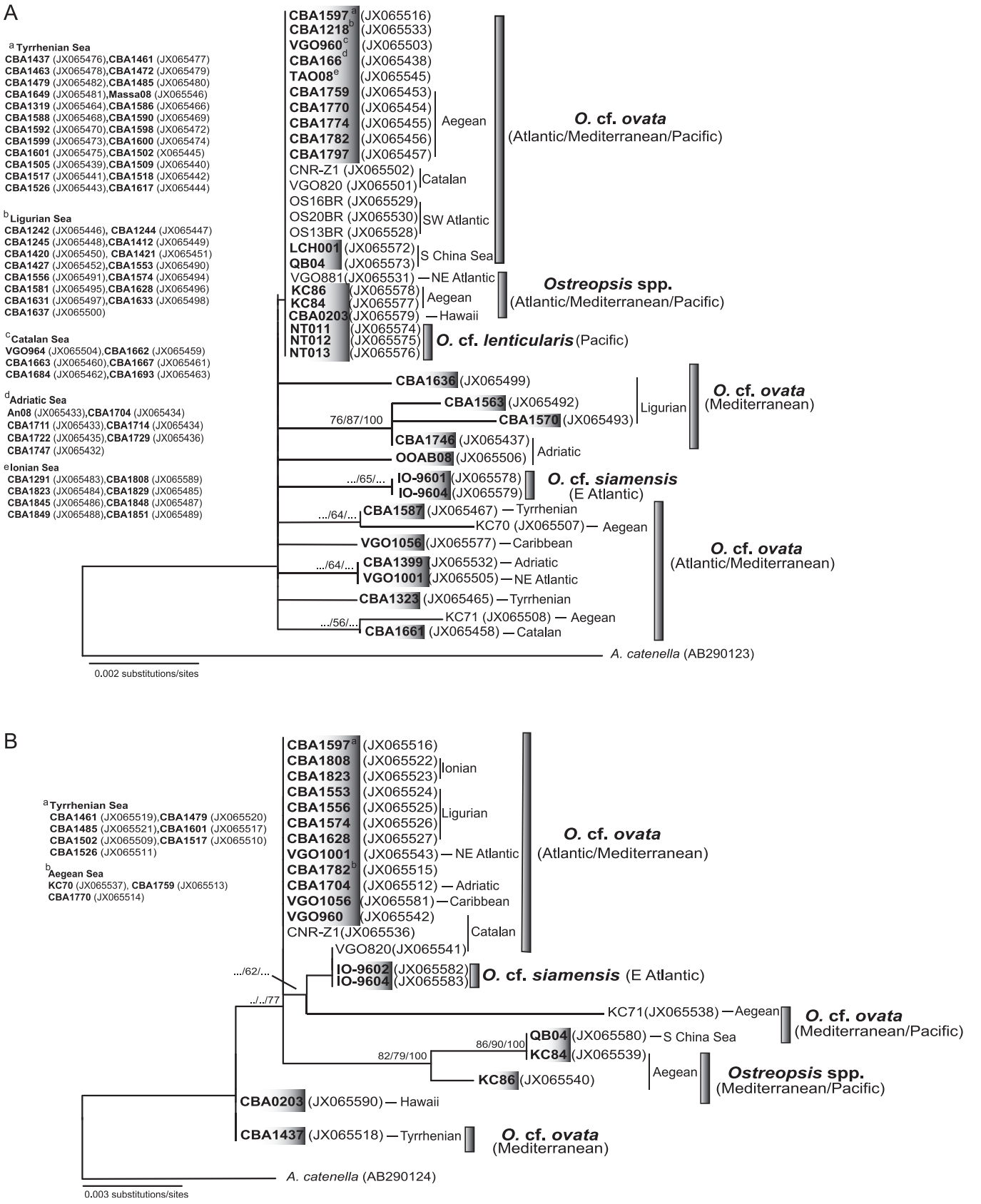


**Fig. 1.** Maximum likelihood phylogenetic tree of the genus *Ostreopsis* inferred from COI (A) and cob (B) mitochondrial gene sequences. The tree is rooted with *Alexandrium catenella* as outgroup. Numbers on the major nodes represent from left to right NJ (1000 pseudo-replicates), ML (1000 pseudo-replicates) bootstrap and Bayesian posterior probability values. Only bootstrap values >50% are shown. Geographical origins of *Ostreopsis* isolates are indicated. All sequences of bold and shaded isolates are obtained in this study.

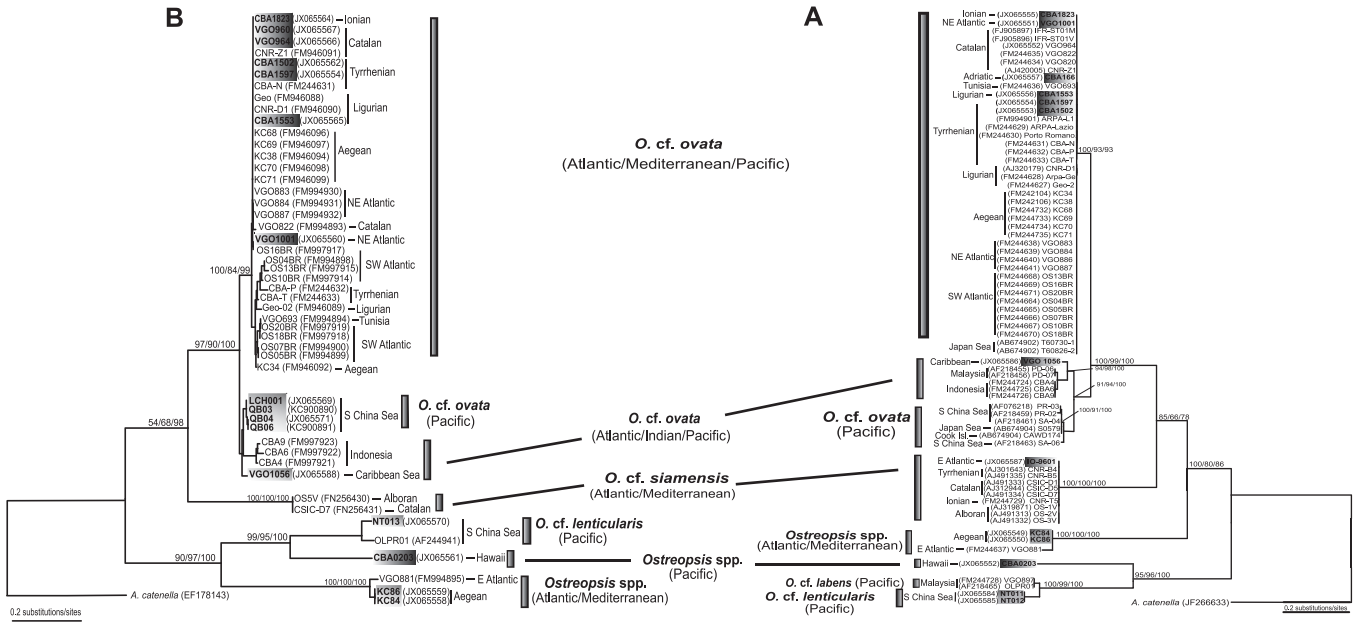


**Fig. 2.** Maximum likelihood phylogenetic tree of the genus *Ostreopsis* inferred from ITS-5.8S (A) and LSU (B) ribosomal gene sequences. The trees are rooted with *Alexandrium catenella* as outgroups. Numbers on the major nodes represent from left to right NJ (1000 pseudo-replicates), ML (1000 pseudo-replicates) bootstrap and Bayesian posterior probability values. Only bootstrap values >50% are shown. Geographical origins of *Ostreopsis* isolates are indicated. All sequences of bold and shaded isolates are obtained in this study. The putative species or clade designations of *Ostreopsis* spp. were shown in the middle also with lines to the corresponding clades in the two phylogenetic analyses. Where it was not possible species name designations were left for each clade.

**Table 3**
Average Pairwise Distance (PWD) expressed as percentage in *Ostreopsis* cf. *ovata* and genus *Ostreopsis* inferred from ribosomal (ITS-5.8S and LSU) and mitochondrial (COI and cob) nucleotide sequences.

| Gene | PWD ± SE in *Ostreopsis* cf. *ovata* | PWD ± SE in genus *Ostreopsis* |
|---|---|---|
| ITS-5.8S | 0.04 ± 0.04 | 22.0 ± 1.3 |
| LSU | 1.2 ± 0.3 | 16.0 ± 1.1 |
| COI | 0.06 ± 0.04 | 0.06 ± 0.05 |
| cob | 0.13 ± 0.06 | 0.19 ± 0.09 |

in 37 isolates. Two major groups of haplotypes were identified: one formed by isolates from the Mediterranean Sea, Atlantic coasts of Spain (Canary Islands), Brazil, and Japan Sea that were distant as many as 10 mutational steps from CBA166. In the second group, isolates from Indo-Pacific areas, with exception of N Atlantic VGO1056, were grouped together being separated by as many as 50 mutational steps from CBA166. A network analysis of *O.* cf. *ovata* haplotypes using LSU rDNA was not performed due to the relative small number of sequenced isolates compared to those whose ITS-5.8S rDNA sequences have been determined.

### 3.5. Intra- and inter-specific variations

Average Pairwise Distance (PWD) was calculated among *Ostreopsis* spp. isolates based on ribosomal genes (ITS-5.8S and LSU) and mitochondrial (COI and cob) to measure the genetic divergence among isolates within a species and among species within the genus *Ostreopsis*. All the four genes considered proved to be highly conserved at species level (Table 3). In particular, within *Ostreopsis* cf. *ovata* the COI gene showed a very low percentage PWD that was similar to ITS-5.8S rDNA PWD value. The cob and LSU gene PWD showed a relative higher variation. In contrast, within the genus *Ostreopsis*, different percentages PWDs were found considering the inter-species level variability. Based on both ribosomal genes of ITS-5.8S and LSU, the percentage PWD was 22 (SE ± 1.3) and 16 (SE ± 1.1), respectively, while the cob and COI genes showed much lower average percentage PWD.

## 4. Discussion

In this study, we investigated the phylogenetic and phylogeographical patterns of toxic dinoflagellate *Ostreopsis* species through the analyses of mitochondrial and ribosomal gene sequence variation by increasing sample collection size worldwide.

The genus is distributed in tropical to temperate coastal waters, with nine different morphospecies described (Parsons et al., 2012). All the original descriptions of *Ostreopsis* species were made according to the international codes of nomenclature based only on morphology, according to the criteria of few taxonomists. However, these criteria used to define the morphospecies vary. As a result, some of the morphologically based *Ostreopsis* species descriptions are not unanimously accepted (GEOHAB, 2012). The original descriptions of *Ostreopsis* spp. were based on wild specimens, but now, it is known that morphological features of *Ostreopsis* cells from clonal cultures are very variable, and some criteria used for describing species are no longer valid (Parsons et al., 2012). There are many available nrDNA sequences of *Ostreopsis* isolates, but none has been obtained from the isolates originally used for the formal description of the nine insofar-described species (Penna et al., 2010; Sato et al., 2011).

In the Mediterranean Sea, *Ostreopsis* sp. is becoming a taxon causing frequent blooms harmful both for humans with toxic aerosols (Crinelli et al., 2012; Vila et al., 2012; Casabianca et al., 2013; Ciminiello et al., 2014) or toxin accumulation in shellfish (Aligizaki et al., 2008) and marine fauna with intoxication or contamination (Tubaro et al., 2011; Amzil et al., 2012; Gorbi et al., 2013). Therefore, it is urgent to accurately define species within genus *Ostreopsis* and to determine their geographical distribution. Molecular phylogenetic analyses proved effective to delineate species boundaries. Insofar this approach has been almost exclusively based on ribosomal markers. However, for most of the organisms the markers of choice are mitochondrial genes, especially those used in the so-called barcoding, that is COI and cob.

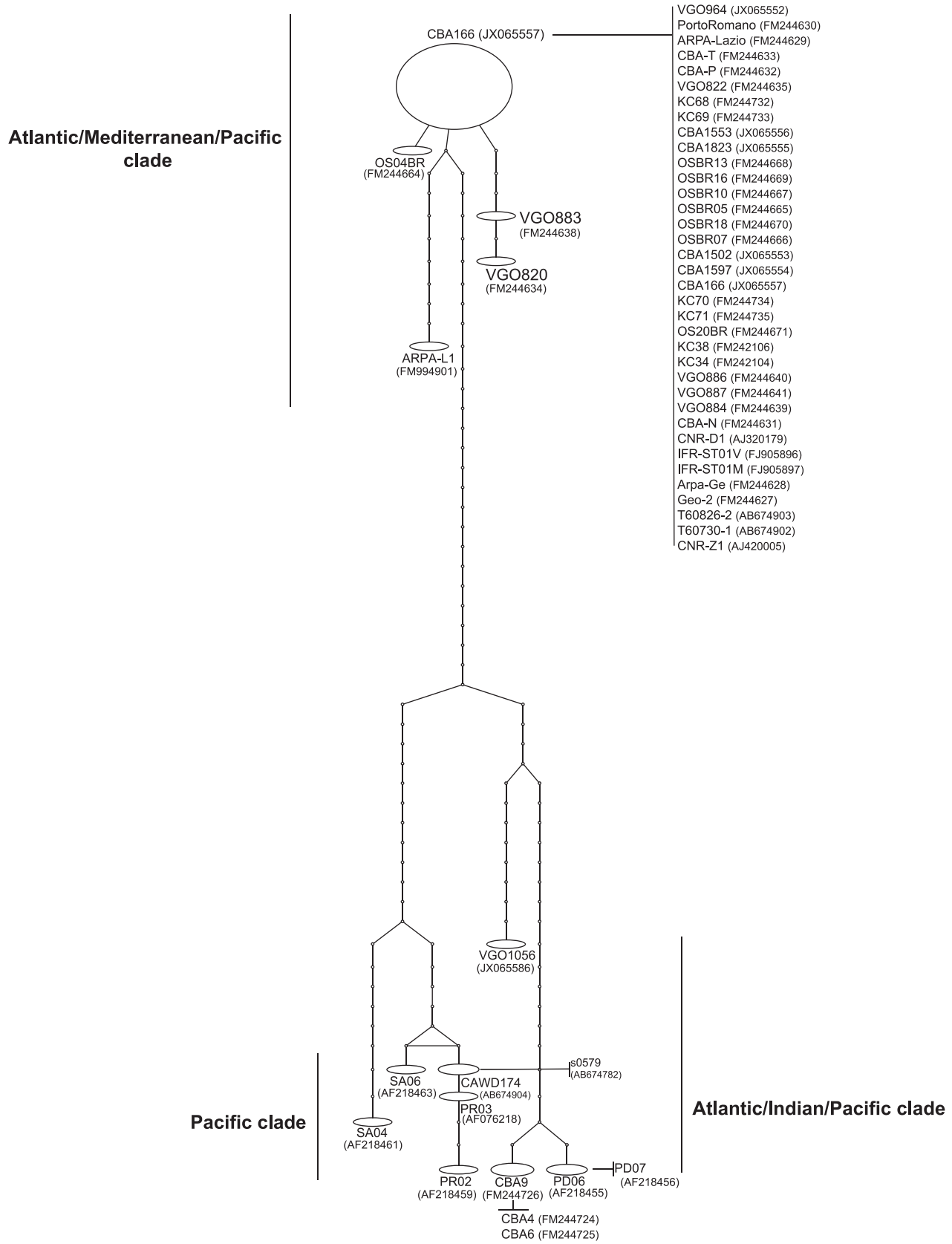


**Fig. 3.** Statistical parsimony network of haplotypes of *Ostreopsis* cf. *ovata* isolates based on ITS-5.8S ribosomal gene sequences. The sizes of the circles are proportional to the number of isolates found having that haplotype and the list of isolates, which share the same haplotype, was shown. Small closed circles indicate missing haplotypes. Geographical origins of *Ostreopsis* cf. *ovata* clades are indicated.

To assess the applicability of mitochondrial genes in dinoflagellates, we provide the first data on COI and cob *Ostreopsis* mtDNA gene sequences. A total of 131 mitochondrial sequences were analyzed. The *Ostreopsis* isolates were collected from several localities, including the (i) Mediterranean Sea, (ii) Brazilian coast, Caribbean Sea, Canary Islands and Portuguese coast in the Atlantic Ocean, (iii) western coast of south China Sea (Vietnam), and (iv) Hawaii Islands in the Pacific Ocean.

The phylogenetic relationships within genus *Ostreopsis* based on COI and cob genes showed that almost all the *Ostreopsis* spp. isolates shared the same nucleotide sequence. None of the two mitochondrial markers analyzed led to any substantial differentiation of various *Ostreopsis* genetic lineages, corresponding also to the different species based on ITS-5.8S and LSU ribosomal sequences. In fact, *Ostreopsis* cf. *ovata*, *O.* cf. *siamensis*, *Ostreopsis* spp. KC84 and KC86 and *Ostreopsis* spp. CBA0203 were grouped together. Further, this high genetic homogeneity among *Ostreopsis* sequences was found despite the wide geographical range of *Ostreopsis* isolate sampling carried out in this study. Therefore, this low level of divergence in both the COI and cob genes indicates they are phylogenetically uninformative and represent inappropriate loci for distinguishing species in this genus. Moreover, the statistical parsimony analysis of *Ostreopsis* spp. isolates using COI and cob genes did not produce any resolution of haplotype clustering based on geographical distribution. A unique main haplotype was generated using both mitochondrial gene sequences (data not shown). The original goal to apply the mtDNA for phylogenetic and phylogeographical analyses within this toxic marine dinoflagellate was feasible. The COI gene, which has been found to be widely applicable in animals, was unsuccessful in most species of plants and fungi due to a much slower rate of evolution compared to animals (Rubinoff et al., 2006; Hollingsworth et al., 2011). Most likely, COI gene has the same feature of a not appropriate evolutionary rate for species level resolution in *Ostreopsis* and most dinoflagellates. COI gene proved to be highly conserved. Most *Alexandrium* species also yielded identical COI sequence, as did *Karenia* spp., *Lingulodinium polyedrum* and *Protoceratium reticulatum* (Lin et al., 2009; Stern et al., 2010). Similarly, little cob sequence variation was observed in *Alexandrium* spp., *Karenia brevis* or *Karenia mikimotoi*. In contrast, the COI and cob gene sequences from dinoflagellate species in the genera *Symbiodinium, Heterocapsa, Prorocentrum*, and *Scripsiella* were sufficiently divergent that they could be used to readily discriminate species (Lin et al., 2009; Stern et al., 2010). In the case of *Prorocentrum minimum* isolates, there was sufficient intraspecific sequence diversity to additionally identify potential population level differences. Therefore, within protists, the applicability of COI and cob gene markers for species discrimination is clearly taxon-specific.

In this study, in contrast, the conserved LSU and 5.8S genes, as well as the more divergent ITS regions, were able to consistently delineate species level divergences among *Ostreopsis* species as already demonstrated by Sato et al. (2011), Penna et al. (2012) and David et al. (2013).

Collectively the rDNA phylogenies indicate the existence of at least eight different species *Ostreopsis* species. Specifically, the ITS phylogeny indicated the existence of the following seven clades: a globally distributed *Ostreopsis* cf. *ovata* (Atlantic/Mediterranean/Pacific), a genetically distinct second globally distributed *O.* cf *ovata* (Atlantic/Indian/Pacific), a third *O.* cf. *ovata* found so far only in the Pacific, *Ostreopsis* cf. *siamensis* (Atlantic/Mediterranean), *Ostreopsis* spp. (Atlantic/Mediterranean), a separate *Ostreopsis* spp. (from Hawaii isolate CBA0203 and sister to the first *Ostreopsis* sp.), *Ostreopsis* cf. *labens* (Pacific) and *Ostreopsis* cf. *lenticularis* (Pacific). The LSU phylogeny which did not contain as complete a sample selection as the ITS (lacks any *O.* cf. *labens* sequence), indicated the presence of seven distinct species. Five of the clades in the two analyses can be confirmed as equivalent because they contain matching sequences from overlapping isolates. The *O.* cf. *ovata* clade from the Pacific and one the *Ostreopsis* spp. (Atlantic/Mediterranean) clades, however, did not contain overlapping sequences from the same isolate and can at this time only be considered potential equivalent species because they are from matching geographic regions (Fig. 2b). The significant genetic differentiation among the different *O.* cf *ovata* clades indicates these might be separate species in the future. However, such species designations should be supported using other robust characters, such as toxin content and profiles or lipids and pigment composition.

There was also evidence from the ITS-5.8S haplotype analysis for three distinct subgroups in the Atlantic/Mediterranean/Pacific *Ostreopsis* cf. *ovata* clade. Each of these subgroups corresponds to isolates originating from distinct geographic regions. The cluster containing the most frequently observed haplotype, CBA166, included isolates from the western and eastern Atlantic coasts and Mediterranean Sea. This geographical group, which comprised also new sequenced isolates, confirmed the previous scenario (Penna et al., 2010). Thus, increasing the sampling size corroborated the hypothesis of a panmictic Atlantic/Mediterranean population. But, this main group now included two isolates from Pacific (Japan Sea). This result was also obtained by Sato et al. (2011): four isolates of Japanese *O.* cf. *ovata* fell into Atlantic/Mediterranean clade based on phylogenetic analysis of ITS-5.8S rDNA. To better address this issue more isolates have to be analyzed from Japan and Atlantic/Mediterranean areas with the same molecular markers. Moreover, more polymorphic markers can be developed and applied to investigate the genetic variation at population level over a defined geographical range (Casabianca et al., 2012). Finally, other comparative analyses based on phenotypic characters (e.g. toxin profile, pigment composition, morphological features) are required to understand the distribution pattern of these isolates.

The other two haplotypes groups of *Ostreopsis* cf. *ovata* comprised isolates deriving from Indo-Pacific areas, mainly Asiatic areas. The exception was for VGO1056 from W Atlantic (Caribbean Sea) that was closer to the Atlantic/Mediterranean/Pacific group (separated by 40 mutational steps) than to the Indo-Pacific group. These two latter groups of *O.* cf. *ovata* might be considered a almost unique and large population of Indo-Pacific Oceans with isolates from distant geographical areas, such as Cook Islands or Malacca Strait. No clear geographical barriers inhibited gene flow among sites. The connection among geographical areas can be mediated by the Equatorial Currents (Sato et al., 2011) explaining the dispersion and the close genetic relationships among the Indo-Pacific isolates over large distances. Anyway, more intense sampling in the Indo-Pcific areas may be needed to fully resolve these slightly population level differences.

Thus, in this study, ribosomal gene sequences sufficiently diverged to allow a phylogenetic and phylogeographical reconstruction within the genus *Ostreopsis*. In contrast, both COI and cob genes lacked such genetic information.

Moreover, we analyzed gene divergence at intra- and inter-specific level based on ribosomal ITS-5.8S and LSU genes or mitochondrial COI and cob genes of *Ostreopsis* species. At the intra-species level, ITS-5.8S rDNA showed very low nucleotide variation considering the *O.* cf. *ovata* percentage PWD values. Similar results were also obtained based on mitochondrial COI and cob genes with very low PWD, while the LSU rDNA showed a relatively higher divergence compared to mitochondrial genes. Nevertheless, PWD measured among *Ostreopsis* species showed a variable degree of divergences based only on ribosomal genes. Despite the greater length of COI and cob genes compared to the ITS regions and LSU D1/D2 gene, the absolute amount of inter-specific variations

within genus *Ostreopsis*, compute as p-distance, are significantly higher for the ribosomal genes, in particular for the ITS-5.8S regions than for mitochondrial ones (Kruskal Wallis Test, $p < 0.0001$).

Thus, in *Ostreopsis* spp. ribosomal nucleotide dataset including the ITS-5.8S and LSU regions we analyzed, it seemed to be evident higher inter-specific genetic variation; while in the mitochondrial gene sequences inter-specific genetic variation is extremely low. Indeed, the mitochondrial gene inter-specific divergence almost equals to the intra-specific variation.

## 5. Conclusions

This study investigated phylogenetic and phylogeographical relationships within genus *Ostreopsis* based for the first time, on mitochondrial and ribosomal genes, with a large worldwide collection of isolates. It was found that mtDNA genes were not able to discriminate the known different species: COI and cob genes resulted too conserved at inter-species level with a presumably low mutation rate. In contrast, the ITS-5.8S and LSU rDNA allowed assigning isolates to known species. *Ostreopsis* cf. *ovata* is a species complex including various clades: the Atlantic/Mediterranean/Pacific clade now includes isolates from Japan Sea, new isolates from Ionian Sea and Tyrrhenian Sea (Mediterranean). The *O.* cf. *ovata* Atlantic/Indian/Pacific clade contains new isolates from Belize. The Pacific clade containes new isolates from Vietnam. The *Ostreopsis* cf. *siamensis* forms an Atlantic/Mediterranean clade. The *Ostreopsis* spp. yet to be taxonomically named include new isolates from Mediterranean and E Atlantic. The *Ostreopsis* cf. *lenticularis*/*O.* cf. *labens* now retains new isolates from Hawaii and Pacific Asia.

To summarize, phylogenetic analysis of ribosomal genes, but not mitochondrial genes appears to provide a robust means of distinguishing *Ostreopsis* species. We anticipate that the additional species will be identified as the taxonomic sampling increases, particularly in geographic regions which are currently unrepresented.

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